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# pH dependence of non-specific adsorption and detection solution in electrochemical metalloimmunoassay using antibody–silver nanoparticle conjugates

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ABSTRACT

The pH dependence of non-specific adsorption of antibody–silver nanoparticle conjugates (AgNPs–Ab) to an antibody-immobilized electrode was investigated. The adsorption was promoted at lower pH values (pH 3.0) and inhibited at higher pH values. These results are attributed to the zeta potential of the conjugates and the electrodes. The zeta potentials of antibody-immobilized graphite powder (as a simulant electrode) were negative above pH 3.5, while those of AgNPs–Ab were negative at more than pH 3.0. Thus, the electrostatic forces between the antibody-immobilized electrode and AgNPs–Ab were attractive in the pH range from 3.0 to 3.5 which the adsorption was promoted. In contrast, above pH 3.5, electrostatic repulsive forces inhibited the adsorption. These investigations suggest that the detection solution above pH 3.5 acts as the washing and the detection solution in an electrochemical metalloimmunoassay. This could reduce the number of steps in the metalloimmunoassay. In the simplified electrochemical metalloimmunoassay using the pH-optimized detection solution, a calibration curve for Hepatitis B surface antigen as a model antigen was obtained. The detection limit was 0.78 IU $mL^{-1}$. The simplified electrochemical metalloimmunoassay, based on AgNPs–Ab and the pH-controlled detection solution, can provide a sensitive and simple detection scheme for various biomarkers in a user-friendly format that is suitable for unskilled users.



## 1. Introduction

Electrochemical immunosensors (EI) have attracted much interest due to the combined advantages of compact size, portability, cost-effectiveness, sensitivity, and speed. EI have a variety of applications in environmental analysis and in clinical diagnostics. In particular, as a result of being compact and portable, EI have a great potential in point-of-care (POC) diagnostics, which can be performed at a bedside or in the clinic to improve the speed and effectiveness of treatment [1–6].

There are two immunoassay formats: heterogeneous and homogeneous assay. The heterogeneous assay has been studied widely, and it has achieved a high sensitivity by using the detection conjugates based on enzyme modified biomolecules [7,8] or nanomaterial conjugates [9–25]. However, the most of those assays require a series of complicated and time-consuming steps: (1) a separation step, which involves solution displacements from a reaction solution including analytes and the detection conjugates to dedicated washing solutions: (2) a washing step: and (3) a detection step, which involves solution displacement from the washing solution to a detection solution such as enzyme substrate and dedicated electrolyte, and signal detection. Therefore, the heterogeneous assay is unlikely to be suitable for unskilled users. Conversely, the homogeneous assay does not require these complicated steps, however, its sensitivity is generally inferior to the heterogeneous assay [26,27].

Nanoparticles are highly beneficial in various applications such as biosensing, biomaterials, and medicine. They have the advantages of their large surface area, stability, ease of preparation, and good biocompatibility. Among them, silver nanoparticle has attracted considerable attention in electro-analytical chemistry due to their low oxidation potential, good catalytic property, and excellent conductivity [28–34].

Biomolecule–nanoparticle conjugates (NPs-conjugates) have been widely used as the detection conjugates in biosensing [35,36] and as the delivery vehicle in medicine [37,38]. There has

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also been growing interests in the interactions between NPs-conjugates and organic or inorganic materials [39–44]. The mechanisms for the adsorption and desorption of NPs-conjugates to and from the biomolecule immobilized surface have been gradually elucidated. Bhan et al. have shown that the electrostatic forces play a central role in the interactions of nanoparticles toward the protein immobilized surface; the electrostatic attractive forces between the negatively charged nanoparticles and the positively charged protein immobilized surface have promoted the significant nanoparticle adsorption [45]. Park et al. have reported that the amount of nanoparticles adsorbed on the surface depends on the pH influencing the electrostatic interactions between them [46]. Shindel et al. have shown that the desorption of nanoparticles from the protein immobilized surface has been attributed to the electrostatic repulsive forces [47]. Here, we considered that this mechanism for the adsorption/desorption can be applied to the EI. For example, it was expected that the non-specific adsorption of NPs-conjugates as the detection conjugates could be suppressed by a pH-controlled washing solution. Furthermore, the number of steps in the heterogeneous metalloimmunoassay could be reduced by a pH-controlled detection solution, which acts as the washing and the detection solution.

In this study, we investigated the pH dependence of non-specific adsorption by antibody–silver nanoparticle conjugates (AgNPs–Ab) to an antibody-immobilized carbon electrode. The zeta potentials of antibody-immobilized graphite powder (as a simulant electrode) and AgNPs–Ab at respective pH were determined by the electrophoretic light scattering method. Finally, we developed a pH-controlled detection solution that inhibits the non-specific adsorption and acts as the washing and the detection solution. Then, we obtained a calibration curve for Hepatitis B surface (HBs) antigen as a model antigen in the simplified electrochemical metalloimmunoassay. Hepatitis B is classified as a serious infectious disease worldwide and the HBs antigen is frequently used to screen for the presence of this virus [48–52]. Therefore, it was chosen as the model antigen to confirm the utility of the simplified electrochemical metalloimmunoassay.

## 2. Materials and methods

### 2.1. Materials

NaCl, KCl, HCl, NaOH, $Na_2HPO_4$, $NaH_2PO_4$ $2H_2O$, Tween 20, and Tris–HCl were purchased from Wako Pure Chemical Industries, Ltd, Osaka, Japan. Polyethylene glycol (PEG, Molecular weight = 20,000) and $KH_2PO_4$ were purchased from Nacalai Tesque, Inc, Kyoto, Japan. The solution of silver nanoparticles, having a 60-nm diameter ($1.7 \times 10^{10}$ particles $mL^{-1}$), and bovine serum albumin (BSA) were purchased from Sigma–Aldrich, St. Louis, Mo, USA. Hepatitis B surface antigen (HISCL HBsAg calibrator), monoclonal primary HBs antibody, and monoclonal secondary HBs antibody were donated by Sysmex Co., Ltd, Kobe, Japan. Planar screen-printed carbon electrode was purchased from BioDevice Technology Ltd, Ishikawa, Japan. Other reagents were of analytical grade, and all solutions were prepared and diluted using ultrapure water (18 MΩ-cm) from a Millipore Milli-Q system.

### 2.2. Preparation of HBs antibody–silver nanoparticle conjugates

The solution containing silver nanoparticles was adjusted to pH 7.0 using 1.0 M HCl. 100 μL of secondary HBs antibody (100 μg $mL^{-1}$ in 10 mM phosphate buffer, pH 7.2) was mixed with 900 μL of silver nanoparticles solution. Then, 10.1 μL of 10% Tween 20 was added to the mixture to prevent the adsorption of antibody–silver nanoparticle conjugates (AgNPs–Ab) to the test tube, and left to stand for 60 min at room temperature. Subsequently, 400 μL of 1% BSA in Tris buffered saline with 0.1% Tween 20 (TBS-T) was added to the mixture for blocking the uncoated surface of silver nanoparticles. Then, AgNPs–Ab were collected by centrifugation (7000*g* for 20 min at 4 °C). After discarding the supernatant, AgNPs–Ab were dispersed in 1 mL of 1% BSA/TBS-T solution. This process was repeated four times. Finally, AgNPs–Ab were dispersed in 200 μL of preservation solution (1% BSA, phosphate buffered saline (PBS), 0.01% Tween 20, pH 7.2).

### 2.3. Fabrication of HBs antigen immunosensor

The primary HBs antibody (which recognizes a different epitope on HBs antigen from AgNPs–Ab) was immobilized on the working carbon electrode by spotting 3 μL of 100 μg $mL^{-1}$ in 50 mM carbonate buffer (pH 8.5) and standing for 2 h at room temperature. Next, unbound antibodies were rinsed with PBS. For the suppression of non-specific adsorption, 4 μL of a blocking solution (3% BSA and 5% PEG in PBS) was spotted on the electrode and left to stand overnight at 4 °C. Finally, the blocking solution was rinsed with PBS.

### 2.4. Immunoreaction

A sandwich-type immunoassay was performed on the working carbon electrode. 2 μL of HBs antigen in fetal bovine serum (FBS) was mixed with 2 μL of AgNPs–Ab solution. Immediately, the mixture was spotted on the electrode and left to stand for 90 min at room temperature in the humidified chamber (at a humidity of 99%).

### 2.5. The pH dependence of non-specific AgNPs–Ab adsorption at washing

After the immunoreaction, the working carbon electrodes were immersed in 200 μL of pH-controlled washing solutions for 10 s with gentle shaking. Subsequently, the electrodes were dried by nitrogen gas. The AgNPs–Ab on the electrodes were observed by means of a field emission scanning electron microscope (FE-SEM) (JSM-7500 FT, JEOL, Japan). Then, the currents were measured using a detection solution of pH 3.0. The pH-controlled washing solutions included 0.05 M chloride ions ($Cl^-$); the pH was adjusted from 3.0 to 10.1 using NaCl, HCl, and NaOH.

### 2.6. Diameter and zeta potential measurements

Graphite powder, which was selected as a simulant electrode, in 50 mM carbonate buffer (pH 8.5) was mixed with the primary HBs antibody (100 μg $mL^{-1}$ in 10 mM PB) and left to stand for 2 h at room temperature. Then, antibody-immobilized graphite powder was collected by centrifugation. After discarding the supernatant, they were dispersed in PBS. After a further centrifugation, antibody-immobilized graphite powder was dispersed in a blocking solution (3% BSA and 5% PEG in PBS). Finally, antibody-immobilized graphite powder was collected by centrifugation, and dispersed in the pH-controlled solution. An aliquot of AgNPs–Ab or silver nanoparticles was mixed with the pH-controlled solution. The zeta potential and the diameter were determined using a Zetasizer Nano ZS instrument (Malvern instruments Ltd, UK). The pH-controlled solutions included 0.05 M chloride ions ($Cl^-$) and their pH was adjusted from 1.4 to 11.7 using NaCl, HCl, and NaOH.

### 2.7. Detection of HBs antigen using a pH-controlled detection solution

After the immunoreaction, the working carbon electrodes were immersed in 200 μL of pH-controlled detection solutions for 10 s

with gentle shaking. Subsequently, electrochemical measurements were performed in 20 μL of the same pH-controlled detection solutions. Thus, the pH-controlled detection solution acted as the washing and detection solution, and the washing step using dedicated washing solutions was not required. The pH-controlled detection solutions included 0.05 M chloride ions ($Cl^-$) and its pH was adjusted from 1.5 to 11.7 using NaCl, HCl, and NaOH.

### 2.8. Electrochemical detection of AgNPs–Ab

The amount of AgNPs–Ab was determined by a sequence of electrical processes. The silver nanoparticles captured on the working carbon electrode were electrically oxidized to silver ions by applying a potential of 2.1 V for 30 s. Then, the silver ions were electrodeposited by applying a potential of −1.0 V for 240 s. Immediately after, the amount of silver electrodeposited on the electrode was determined using differential pulse voltammetry (DPV). Instrumental parameters of the DPV were as follows: the potential was 4 mV, the pulse amplitude was 50 mV, and the pulse period was 0.2 s. Electrochemical measurements were performed using a potentiostat (Model 832b, BAS Inc.).

## 3. Results and discussion

We expected that the electrostatic forces between antibody-immobilized electrode (Ab-electrode) and antibody–silver nanoparticle conjugates (AgNPs–Ab) would vary between attractive and repulsive depending on the pH of the washing solution. Thus, if the pH induced electrostatic attractive forces between them, the non-specific AgNP–Ab adsorption was promoted at washing step, which would increase the noise. Conversely, in the case of electrostatic repulsive forces, the non-specific adsorption was inhibited, which would decrease the noise.

### 3.1. pH dependence of non-specific AgNPs–Ab adsorption

Firstly, we investigated the pH dependence of non-specific AgNPs–Ab adsorption at washing step using FE-SEM and electrochemical techniques. We derived the particle density of AgNPs–Ab on the electrode for 0 IU $mL^{-1}$ and 50 IU $mL^{-1}$ HBs antigen from FE-SEM images (Fig. 1a). The densities obtained for 50 IU $mL^{-1}$ HBs antigen were almost constant regardless of the pH. In contrast, the densities due to non-specific adsorption obtained for 0 IU $mL^{-1}$ tended to be largest at pH 3.0, although the difference between the densities at respective pH values was small. With FE-SEM, it was not difficult to show the entire electrode (~2.8 $mm^2$); only localized areas (~46 $\mu m^2$) were observed. Therefore, we measured oxidation currents for 50 IU $mL^{-1}$ HBs antigen (signal) and 0 IU $mL^{-1}$ HBs antigen (noise) using the electrodes observed by FE-SEM (Fig. 1b). The signals were almost constant regardless of the pH, which reflects the constant AgNPs–Ab amount on the electrodes as shown in Fig. 1a. In contrast, the noise was dependent on the pH; noise was suppressed at the higher pH value. Therefore, we conclude that the amount of non-specific AgNPs–Ab adsorption depends on the pH at washing step in an electrochemical metalloimmunoassay.

### 3.2. pH dependence of zeta potential and diameter for respective materials

Then, we confirmed that the pH dependence of non-specific AgNPs–Ab adsorption was attributed to the electrostatic forces by measuring the zeta potential of antibody-immobilized graphite powder (as a simulant electrode) and AgNPs–Ab (Fig. 2a). The zeta potential of the simulant electrode was neutral (iso-electric point: IEP) at around pH 3.5, positive at a lower pH value, and negative at a higher pH value. It shifted positively (by about 6 mV) in the measured pH range, in comparison with that for graphite powder. We consider that the shift is likely to be attributed to BSA and antibody (IEP of BSA is around 4.7 and that of antibody is around 7.0) immobilized on the graphite. The zeta potential of AgNPs–Ab was negative more than pH 3.0. It also shifted positively in comparison with that for silver nanoparticles, which were about −70 mV in the pH range from 5.0 to 10.0. The shift is also likely to be attributed to BSA and antibody on the silver nanoparticles. In fact, the diameters of AgNPs–Ab were larger than those of silver nanoparticles in the pH range from 5.0 to 10.0 (Fig. 2b). This shows that silver nanoparticles were conjugated to antibodies successfully. However, we could not measure the zeta potentials of silver nanoparticles accurately in the lower or higher pH range, due to the aggregation of silver nanoparticles. In the pH range from 1.0 to 2.0, the zeta potential of AgNPs–Ab fluctuated, and the diameter of AgNPs–Ab was comparable to silver nanoparticles. This probably shows that antibodies started to be released from silver nanoparticles; subsequently, silver nanoparticles started to aggregate. Thus, in the pH range from 1.0 to 2.0, AgNPs–Ab were unstable and the zeta potentials could not be measured accurately. Above pH 3.0, the zeta potential of AgNPs–Ab gradually decreased as pH increased. The decrease was attributed to the deprotonation of the carboxyl groups on antibodies or BSA on silver nanoparticles [45–47]. The zeta potential measurements showed that electrostatic repulsive forces between Ab-electrode and AgNPs–Ab resulted above pH

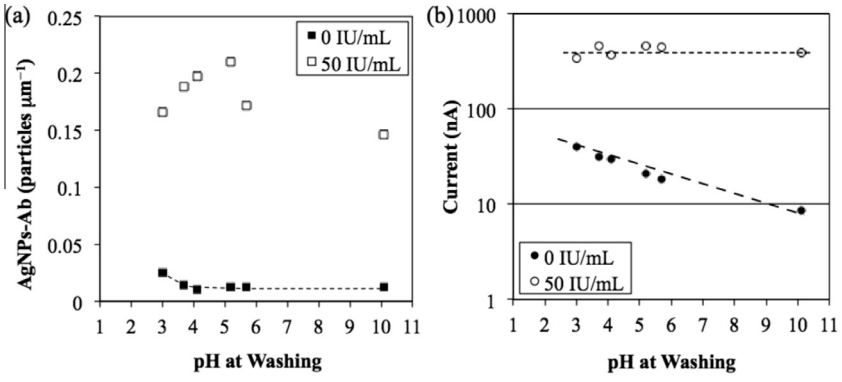


**Fig. 1.** (a) The pH dependence of the particle density (particles $\mu m^{-2}$) of AgNPs–Ab on the electrode treated with washing solution of pH 3.0, 3.7, 4.1, 5.2, 5.7, 10.1 after the immunoreaction in the absence (0 IU $mL^{-1}$) or in the presence of HBs antigen (50 IU $mL^{-1}$). The particle densities were derived from the average of 15 areas on the same electrode observed by means of FE-SEM. (b) The pH dependence of corresponding currents of (a). Current measurements were performed using a detection solution of pH 3.0. The horizontal axis shows the pH at washing step before observations by means of FE-SEM.

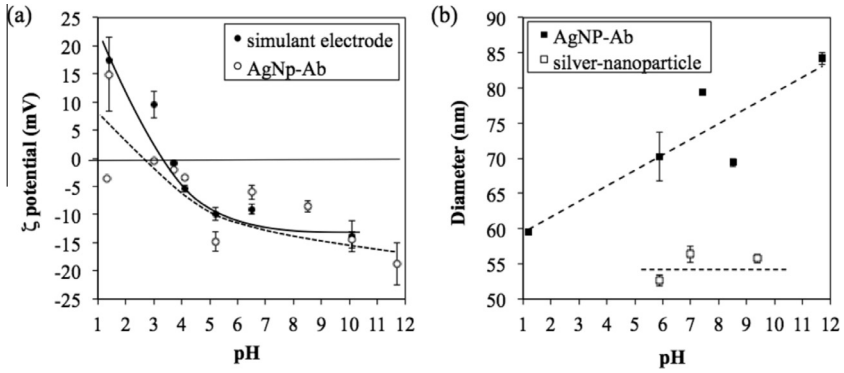


**Fig. 2.** The pH dependence of (a) the zeta potential for antibody-immobilized graphite powder (simulant electrode) and AgNPs–Ab determined by the electrophoretic light scattering method, and (b) the diameter for AgNPs–Ab and silver nanoparticles determined by the dynamic light scattering method. The data points and error bars correspond to the average and the standard deviation from three independent measurements.

3.5, since the zeta potentials of these were likely to be negative. Then, the electrostatic attractive forces resulted in the pH range from 3.0 to 3.5, since the zeta potential of the Ab-electrode was likely to be positive while that of AgNPs–Ab was negative. Thus, in the case of using the washing solution in the pH range from 3.0 to 3.5 at washing step, the non-specific AgNPs–Ab adsorption was promoted by the electrostatic attractive forces. In contrast, in the case of using the washing solution above pH 3.5, it was inhibited by the electrostatic repulsive forces. Moreover, the amount of non-specific adsorption was suppressed at higher pH values as shown in Fig. 1b, since the electrostatic repulsive forces were strengthened.

### 3.3. Optimization of pH-controlled detection solution

The washing step and the detection step using respective dedicated solutions have been performed in the conventional electrochemical metalloimmunoassay as follows (Fig. 3a); after the sandwich-type immunoassay using AgNPs–Ab, the washing steps were performed using dedicated washing solutions. Then, after the solution displacement and drying process, the detection steps such as the electrical pretreatment and current measurement were performed using a dedicated detection solution.

However, it was expected that the number of step before the signal detection could be reduced by applying the insight from the above pH dependence of non-specific adsorption and by developing the pH-controlled detection solution acting as the washing and the detection solution. In other words, as shown in Fig. 3b, the washing and detection steps are performed using the same pH-controlled detection solution. Therefore, the washing steps using the dedicated washing solutions are not required in the simplified electrochemical metalloimmunoassay, so that the complicated steps such as the solution displacements and drying process are not required and the time until the signal detection becomes shorter than it for the conventional one shown in Fig. 3a. Then, we examined the pH dependence of the intensity of current obtained in the absence (noise) and in the presence of HBs antigen (signal), and derived signal-to-noise ratio (S/N) in the simplified electrochemical metalloimmunoassay shown in Fig. 3b. The noise dramatically decreased above pH 3.0 (Fig. 4a), due to the electrostatic repulsive forces between Ab-Electrode and AgNPs–Ab as seen from Fig. 2a. The signal gradually decreased

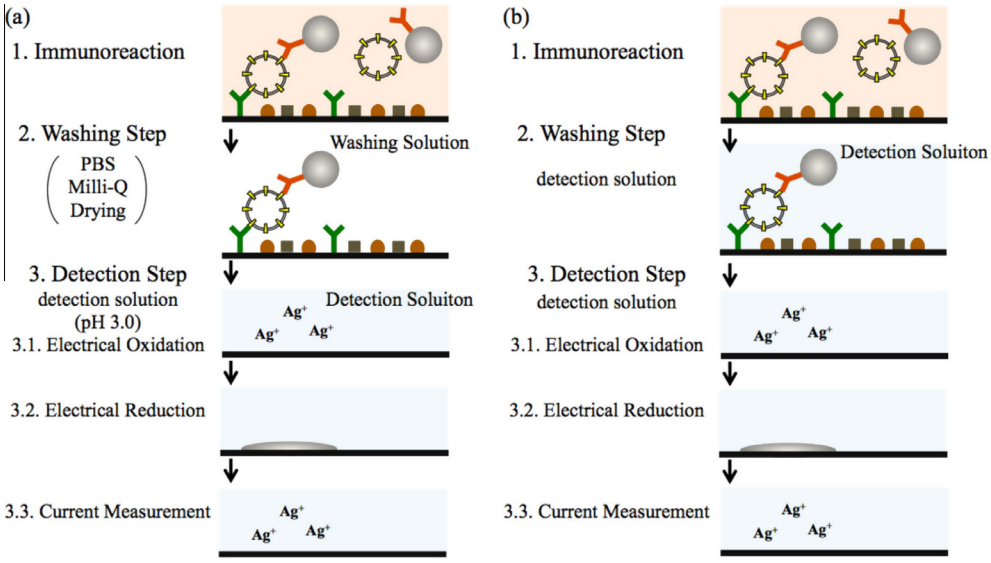


**Fig. 3.** Procedure diagram for (a) the conventional electrochemical metalloimmunoassay, which performed the formation of sandwich-type immunocomplex using AgNPs–Ab, washing steps using dedicated washing solutions and a blower, solution displacement, and detection step using a detection solution; and (b) the simplified electrochemical metalloimmunoassay, which performed the formation of sandwich-type immunocomplex using AgNPs–Ab, and the washing and the detection step using a same pH-controlled detection solution [31].

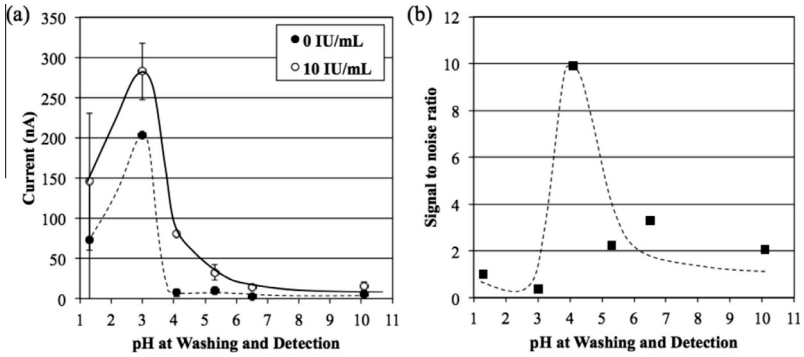


**Fig. 4.** The pH dependence of (a) the intensities of the currents obtained for 0 IU $mL^{-1}$ and 10 IU $mL^{-1}$ HBs antigen in the simplified electrochemical metalloimmunoassay using the AgNPs–Ab and respective pH-controlled detection solutions. The data points and error bars correspond to the average and the standard deviation from three independent measurements. (b) Signal-to-noise ratio derived from (a).

at higher pH values. The pH dependence of signal was not attributed to the different amount of AgNPs–Ab captured on the electrode. The dependence is more likely a result of the potential difference applied to the working electrode, due to the different pH detection solution. In terms of the signal dependence on pH, further investigations are required. The difference in the intensity of noise at pH 3.0 between Fig. 1b and Fig. 4a is attributed to the different steps in measuring the currents. In the case of Fig. 1b, the solution remaining on the electrode after the washing step was removed completely by nitrogen gas. However, in the case of Fig. 4a, it was not removed completely, as shown in Fig. 3b. Thus, the small amount of AgNPs–Ab in the remaining solution most likely induced the difference in the intensity of noise at pH 3.0. In the simplified electrochemical metalloimmunoassay, the S/N was maximum at pH 4.1. We infer that the electrostatic forces between Ab-electrode and AgNPs–Ab varied from attractive to repulsive around pH 3.5, and electrostatic repulsive forces inhibited the non-specific AgNPs–Ab adsorption, which suppressed the noise.

### *3.4. Performance assessment of simplified electrochemical metalloimmunoassay*

Finally, the analytical sensitivity of the simplified electrochemical metalloimmunoassay using AgNPs–Ab was determined using various concentrations of HBs antigen in FBS. Moreover, the sensitivity was compared with that determined in the conventional electrochemical metalloimmunoassay. The currents obtained from the simplified electrochemical metalloimmunoassay are shown in Fig. 5a. In the case employing a solution of pH 4.1 as a detection solution, the detection limit for HBs antigen was found to be 0.78 IU $mL^{-1}$, which was estimated from three times the standard deviation for 0 IU $mL^{-1}$. The standard deviation for 0 IU $mL^{-1}$ was calculated from three measurements using three independently prepared electrodes. In contrast, the detection limit in the conventional metalloimmunoassay, performing solution displacements, washing steps using PBS, Milli-Q and a blower, and a detection step using a detection solution of pH 3.0 [31], was 0.5 IU $mL^{-1}$ (Fig. 5c); this is comparable with the commercial enzyme linked immunosorbent assay (ELISA) kit [51,53]. The detection limit in the simplified electrochemical metalloimmunoassay was 1.6 times higher than the conventional one; however, this approach eliminates the complicated and time-consuming steps such as solution displacements and washing steps using dedicated washing solutions. Therefore, it is expected to be user-friendly and suitable for unskilled users, thus ensuring a potential in POC diagnostics.

## 4. Conclusions

We investigated the pH dependence of non-specific adsorption by AgNPs–Ab in an electrochemical metalloimmunoassay. The pH dependence was attributed to electrostatic forces between antibody-immobilized electrode (Ab-electrode) and AgNPs–Ab. Moreover, we reduced the number of complicated and

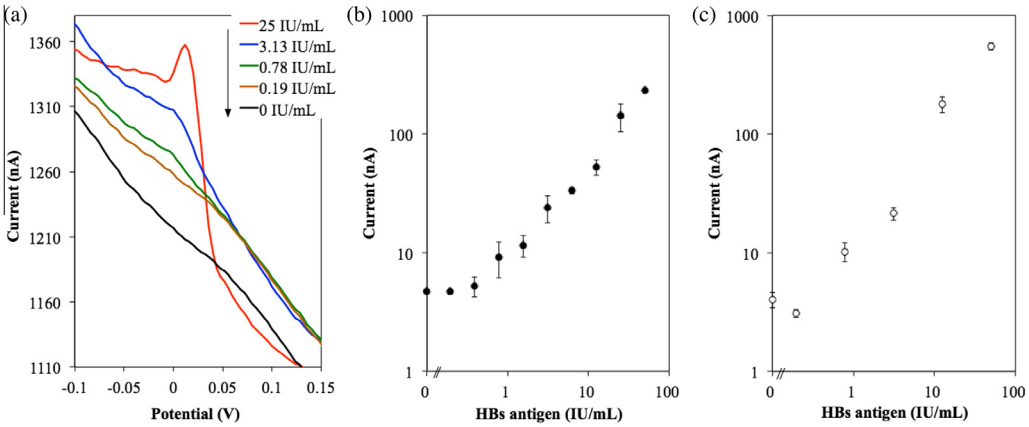


**Fig. 5.** (a) Differential pulse voltammograms obtained in the simplified electrochemical metalloimmunoassay using AgNPs–Ab and a detection solution of pH 4.1. The concentration of HBs antigen was 25, 3.13, 0.78, 0.39, and 0 IU $mL^{-1}$. (b) Calibration plot of HBs antigen obtained from the intensities of the currents which derived from the difference between peak value and baseline. (c) Calibration plot of HBs antigen obtained in the conventional electrochemical metalloimmunoassay using AgNPs–Ab, washing solutions, a blower, and a detection solution of pH 3.0. The data points and error bars correspond to the average and the standard deviations from three independent measurements.

time-consuming steps in the electrochemical metalloimmunoassay successfully by developing a pH-controlled detection solution acting as the washing and the detection solution. The detection limit in the simplified electrochemical metalloimmunoassay was slightly higher than the conventional one that involves the complicated steps such as solution displacements, washing steps using dedicated washing solutions and drying process. In the simplified electrochemical metalloimmunoassay, however, the complicated steps are not required. As such, our approach provides a sensitive and simple detection method for various biomarkers in a user-friendly format that is suitable for unskilled users.

## Conflict of interest

The authors have no conflict of interest directly relevant to the content of this article. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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